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Genetic Resources

Construction and Testing of an Intron-Containing Luciferase Reporter Gene From *Renilla reniformis*

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Abstract. We describe a new *Renilla reniformis* luciferase reporter gene, *RiLUC*, which was designed to allow detection of luciferase activity in studies involving *Agrobacterium*-based transient expression studies. The *RLUC* gene was altered to contain a modified intron from the castor bean catalase gene while maintaining consensus eukaryotic splicing sites recognized by the plant spliceosome. *RLUC* and *RiLUC* reporter genes were fused to the synthetic plant SUPER promoter. Luciferase activity within agrobacteria containing the SUPER-*RLUC* construct increased during growth in culture. In contrast, agrobacteria harboring the SUPER-*RiLUC* gene fusion showed no detectable luciferase activity. Agrobacteria containing these gene fusions were cotransformed with a compatible normalization plasmid containing a cauliflower mosaic virus 35S promoter (CaMV) joined to the firefly luciferase coding region (*FiLUC*) and infused into tobacco leaf tissues through stomatal openings. The kinetics of luciferase production from the *RLUC* or *RiLUC* reporters were consistent, with expression of the *RiLUC* gene being limited to transiently transformed plant cells. *RiLUC* activity from the reporter gene fusions was measured transiently and within stably transformed tobacco leaf tissues. Analysis of stably transformed tobacco plants harboring either reporter gene fusion showed that the intron altered neither the levels of luciferase activity nor tissue-specific expression patterns driven by the SUPER promoter. These results demonstrate that the *RiLUC* reporter gene can be used to monitor luciferase expression in transient and stable transformation experiments without interference from contaminating agrobacteria.

Key words: dual luciferase assay, firefly, intron, luciferase, *Photinus pyralis*, *Renilla reniformis*, reporter gene, sea pansy

Abbreviations: CaMV 35S, cauliflower mosaic virus 35S RNA promoter; DTT, dithiothreitol; *GUS*, β-glucuronidase; Km, kanamycin; LB, Luria-Bertani; *FLUC*, *Photinus pyralis* (firefly) luciferase; *FiLUC*, *Photinus pyralis* (firefly) intron-modified luciferase; LARII, luciferase reagent II (Promega); $MgCl_2$, magnesium chloride; MS, Murashige and Skoog; ng, nanograms; Nos^t, nopaline synthase terminator; *NPTII*, neomycin phosphotransferase II; pBS, pBluescript II (SK+); S&G, stop and glo (Promega); R_0, primary transformant; R_1, progeny of self-fertilized R_0 plant; *RLUC*, *Renilla reniformis* (sea pansy) luciferase; *RiLUC*, *Renilla reniformis* (sea pansy) intron-modified luciferase; RLU, relative light units; TBE, tris-borate EDTA; T-DNA, transferred-DNA.

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Introduction

Reporter genes such as β-glucuronidase (*GUS*) (Jefferson et al., 1987), green fluorescent protein (*GFP*) (Leffel et al., 1997), firefly (*Photinus pyralis*) luciferase (*FLUC*) (Ow et al., 1986), and sea pansy (*R. reniformis*) luciferase (*RLUC*) (Mayerhofer et al., 1995) have become an integral part of plant molecular research. They have become instrumental in studying gene silencing, intron splicing, *Agrobacterium* T-DNA transfer, gene regulation, and both transcriptional and posttranscriptional regulatory processes. They have also proved useful in identifying mutations associated with novel plant phenotypes.

In many cases, the most efficient mechanism for transforming test gene constructs into plants involves the use of agrobacteria, which are often able to ectopically express the plant reporter gene at levels that interfere with accurate measurement of reporter gene activity in planta. Agrobacteria can persist in primary and secondary stable transformants, producing an unacceptable level of background reporter gene activity. This can be overcome by modifying reporter genes to contain a plant intron that only eukaryotic cells are able to efficiently remove from primary transcripts to generate functional mRNA. *GUSi* (Ohta et al., 1990), GUS^{INT} (Vancanneyt et al., 1990), and *FiLUC* (Mankin et al., 1997) reporter genes were successfully modified to include plant introns. The resulting intron-containing genes prevented functional mRNA synthesis in *Agrobacterium* while being spliced efficiently in plants to generate translationally competent transcripts (Ohta et al., 1990).

While developing an agrobacteria-based, in vivo, dual luciferase (*FLUC*, *RLUC*) assay (Cazzonelli, manuscript in preparation), the design and testing of a plant-functional intron-containing version of the *RLUC* reporter gene (*RiLUC*) was necessary to provide an internal control for normalizing *FiLUC* activity in plant cells. A modified version of the castor bean catalase gene (*CAT-1*) intron, used in the construction of *GUSi* (Ohta et al., 1990), was chosen as the portable intron because of its low homology with the PIV2 intron contained within the *FiLUC* reporter gene (Mankin et al., 1997). This report describes the engineering and testing of the *RiLUC* reporter gene. Under control of the SUPER promoter, *RiLUC* expressed luciferase activity in tobacco cells but not in *A. tumefaciens*. Functional properties of the native *RLUC* reporter gene were maintained.

Materials and Methods

Construction of an intron-containing RiLUC *reporter gene*

Standard cloning techniques (Sambrook and Russell, 2001) were used during reporter gene and binary vector construction. pE1778-*RLUC* (Figure 1A) was prepared by amplifying the *RLUC* gene from the pRL-null vector (Promega) by using PCR oligonucleotides that incorporated *Kpn* I and *Xba* I sites at the ends of the PCR product and cloning into pUC19. *RLUC* was excised from pUC19 with

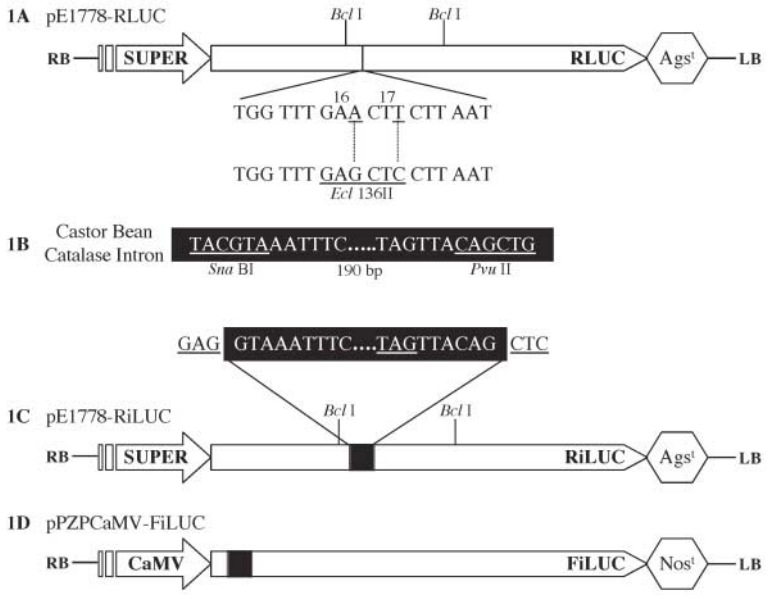

Figure 1. Schematic diagram showing the construction of the intron-modified sea pansy luciferase (*RiLUC*) reporter gene and binary constructs. (1A) Diagrammatic figure of pE1778-*RLUC* showing the SUPER promoter fused to the *RLUC* gene. Nucleotides in codons 16 and 17 that were mutated to generate an *Ecl* 136II site are underlined. (1B) Flanking sequences of the castor bean catalase intron. (1C) Schematic figure of pE1778-*RiLUC* showing flanking sequences of the modified *RiLUC* reporter gene. (1D) Figure shows a CaMV35s–driven firefly luciferase reporter gene fusion. RB, right T-DNA border; LB, left T-DNA border; Ags^t, agropine synthase terminator; Nos^t, nopaline synthase terminator; *RLUC*, *Renilla reniformis* luciferase reporter gene; *RiLUC*, intron-modified *R. reniformis* luciferase reporter gene; *FiLUC*, firefly luciferase reporter gene.

Kpn I and *Xba* I restriction endonucleases and cloned into compatible sites downstream of the synthetic SUPER promoter (Ni et al., 1995) contained within the binary vector pGPTV (Becker et al., 1992). An *Ecl* 136II restriction site was introduced into the *RLUC* gene by silent PCR mutagenesis at codons 16 and 17 by using a primer set designed to alter the region between nearby *Bcl* I restriction enzyme sites (Figure 1A). The amplified PCR product was digested with *Bcl* I and cloned in frame into the *RLUC* gene. A modified version of the castor bean catalase gene intron used in *GUSi* (Ohta et al., 1990) was amplified by means of PCR with primers introducing *Sna* BI and *Pvu* II sites on the 5′ (*Sna* BI) and 3′ (*Pvu* II) ends of the intron (Figure 1B). The amplified product was digested with *Sna* BI and *Pvu* II restriction endonucleases and cloned into the *Ecl* 136II restriction site (Figure 1C). The resulting gene was designated *RiLUC* and sequenced to confirm the orientation of the intron and to ensure that consensus eukaryotic splicing sites recognized by the plant spliceosome were generated during cloning procedures.

In addition to the above binary vectors, a normalization binary plasmid (pPZP200) (Hadjdukiewicz et al., 1994) harboring an intron-modified firefly luciferase gene (Mankin et al., 1997) driven by the enhancer region (–99 to –299 bp)

of the CaMV35S promoter fused to a CaMV 35S minimal domain (+1 to –46 bp) (Benfey and Chua, 1990) was constructed (Figure 1D).

Agrobacterium-*mediated transformation of tobacco*

Binary plasmids were electroporated into EHA105 electrocompetent *Agrobacterium* cells by using methods described by Walkerpeach and Velten (1994). *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabaccum*, cv SR1) was carried out by means of the leaf disc method (Horsch et al., 1988; Svab et al., 1995). Regenerated plants were maintained on MS-agar medium containing B5 vitamins, 100 µg/mL kanamycin sulfate, and 500 µg/mL Claforan (sodium cefotaxime, Hoechst). Primary transformants that rooted on selective media were transferred to seedling mixture, maintained under humid conditions for a week, potted in a sandy/peat soil mixture, and grown in an environmentally-controlled glasshouse (70% humidity, 22°C). Flowering plants (R_0) were self-fertilized and covered to prevent cross contamination. First generation (R_1) seeds were harvested. The transgenic nature of independent lines was confirmed by means of luciferase assays.

Luciferase detection in agrobacteria

Agrobacteria harboring pE1778-*RLUC* and pE1778-*RiLUC* binary vectors were grown at 28°C overnight in LB media (Sambrook and Russell, 2001) containing 25 µg/mL kanamycin selection. We used 200 µL of the starter culture to inoculate 4.8 mL of YEB growth media containing 25 µg/mL kanamycin sulfate. Optical density (OD_{600}) and luciferase activity were measured every hour for 12 h. We injected 25 µL of 14.16 µM coelenterazine (Calbiochem, prepared in DMSO) into a 96-well luminescence plate (polyfiltronics) containing 200 µL of agrobacteria cell suspension. Relative light units (RLUs) emitted were measured for 60 s by using a FLUOstar Optima luminometer (BMG lab technologies). Background levels of light emission were determined by using LB growth media containing 25 µg/mL kanamycin selection and 14.16 µM coelenterazine and subtracted from sample measurements.

Luciferase detection in tobacco leaf tissues infused with agrobacteria

Agrobacteria harboring pE1778-*RLUC* and pE1778-*RiLUC* binary plasmids were transformed with a compatible binary vector containing a CaMV35S promoter–*FiLUC* fusion. Agrobacteria were grown at 28°C in LB media containing 25 µg/mL kanamycin sulfate and 100 µg/mL spectinomycin selection until an OD_{600} of 0.8 was reached. Tobacco plants were grown in hydroponics solution under artificial lighting with a 16/8 photoperiod in an environmentally-controlled growth room (21°C). *Agrobacterium*-mediated transient transformation was conducted on young expanded leaves still attached to the plant. Bacterial suspensions were washed in infiltration media (50 mM MES, 0.5% glucose, 2 mM $NaPO_4$, 100 µM Acetosyringone), resuspended, and infused into tobacco leaf tissues through stomatal openings by pressing the tip of a 1-mL plastic syringe against the lower leaf surface and applying gentle pressure. Six leaf discs for each treatment were frozen in liquid nitrogen 68 h after agro-infusion and stored at –80°C.

Luciferase activity in infused leaf tissues was measured using an in vitro dual luciferase assay system (Promega). Leaf discs were ground in 300 µL of passive lysis buffer (Promega), incubated for 1 h at 4°C, and centrifuged for 10 min at 12000xg. The bioluminescent assay was started by automatically injecting 50 µL of luciferase reagent II (Promega) into 10 µL of cell lysate. A FLUOstar Optima luminometer (BMG Lab Technologies) was used to take 5-s measurements. Then, 50 µL of stop and glo reagent was added to the reaction. A 5-s measurement of Renilla luciferase luminescence was started after a 2.5-s delay. For each experiment, background luciferase activity from noninfected leaf tissue was subtracted. RLUs determined for the *RLUC* and *RiLUC* treatments were normalized to light values determined for the firefly luciferase gene, serving as an internal control.

Luciferase detection in stable transformed tobacco

R_0 transformants harboring pE1778-*RLUC* and pE1778-*RiLUC* promoter-reporter gene fusions were assayed by means of the in vivo leaf disc assay (C. Cazzonelli, in preparation). Duplicate leaf discs from transformants that survived transgenic selection were removed from the third or fourth true leaves using a sharp cork borer. Discs were handled so as to avoid additional wounding of the tissue. Discs were floated on coelenterazine substrate buffer (11.8 µM coelenterazine dissolved in methanol [Calbiochem], 25 mM glucose, 0.1 M $NaPO_4$ [pH 8], 0.05 M NaCl, 1 mM EDTA, 10 mM DTT) in a 96-well white plate. Photons emitted per second were measured over a 2-h incubation period (FLUOstar Optima luminometer, BMG Lab Technologies). Background luminescence from sea pansy luciferase assays was determined by floating leaf discs from wild-type SR1 tobacco on coelenterazine substrate buffer. RLUs were adjusted by subtracting background luminescence from the maximum levels of light emission determined for each assay.

R_1 transgenic tobacco seeds were grown in seedling mixture under artificial light with a photoperiod of 16/8 h in an environmentally-controlled growth room (21°C). After 3-4 wk of vegetative growth, duplicate leaf discs were removed from the third or fourth true leaves from 4-8 individual plants and assayed as described. Average RLUs of 4-8 homozygous/heterozygous plants from 19 independent transformants were determined. Bioluminescence was visualized in vivo by using a Photometrics Cool SNAP HQ imaging system (Roper Scientific). Transgenic seeds were germinated on MS-agar medium containing B5 vitamins. Seedlings were submerged in an 11.8-µM solution of coelenterazine dissolved in pure H_2O and vacuum infiltrated for 1 min. After 2 min of incubation in the dark, 10 min of photon counting ensued.

Results and Discussion

Construction of an intron-containing luciferase gene (RiLUC)

A new reporter gene (*RiLUC*) containing a portable intron within the *R. reniformis* luciferase coding sequence was constructed (Figure 1). A modified intron from the *CAT-1* gene used in the construction of *GUSi* (Ohta et al., 1990) was inserted into an *Ecl136* II restriction site generated by means of PCR mutagenesis

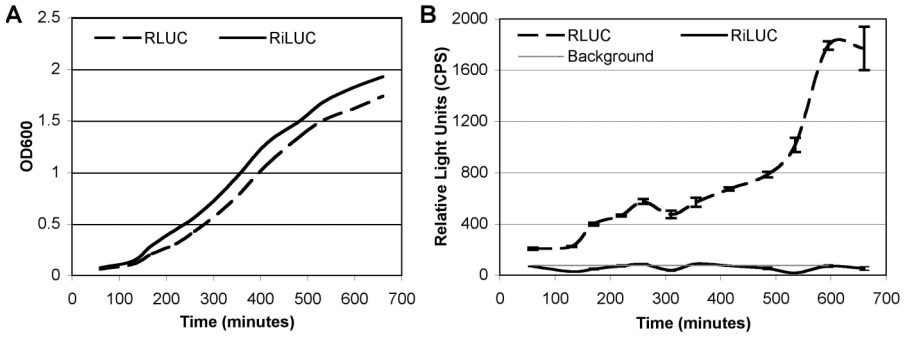

Figure 2. Luciferase activity in agrobacteria. (A) Optical densities (600 nm) of agrobacteria harboring pE1778-*RLUC* (*RLUC*) and pE1778-*RiLUC* (*RiLUC*) binary plasmids. (B) Relative light units were measured by using a FLUOstar Optima luminometer for 60 s after adding coelenterazine substrate to agrobacteria. Error bars represent the standard error of 8 replicate treatments.

of wobble positions 318/321 in codons 16/17 of the *RLUC* gene (Figure 1A, 1B). The sequences at the splice junctions (AG|GTAAA…TACAG|CT) of *RiLUC* are almost identical to those in GUS^{INT} (Vancanneyt et al., 1990) and luc^{INT} (Mankin et al., 1997) optimized with respect to eukaryotic splicing sequences recognized by the plant spliceosome (Shapiro and Senepathy, 1987). The 190-bp modified CAT-1 intron has a 73% AT content and contains multiple stop codons in all translational reading frames that prevent translational read-through.

Analysis of RLUC *and* RiLUC *in* A. tumefaciens

RLUC and *RiLUC* reporter genes were fused to the synthetic plant SUPER promoter (Ni et al., 1995) contained within the binary vector pGPTV (Becker et al., 1992) and transformed into *Agrobacterium* strain EHA105. *RLUC* and *RiLUC* luciferase activity was analyzed in vivo to test if the intron prevents luciferase production in agrobacteria cells. As expected, agrobacteria containing the *RiLUC* reporter gene showed no luciferase activity during growth stages (Figure 2). In contrast, agrobacteria harboring the *RLUC* reporter gene showed increased luciferase activity with increased agrobacteria cell density (Figure 2). We conclude that the agrobacteria failed to splice the CAT-1 intron from the *RiLUC* reporter gene. This suggests that the CAT-1 intron efficiently discriminates between agrobacteria and plant gene expression as was observed during *GUSi* construction (Ohta et al., 1990).

Transient expression of RLUC *and* RiLUC *in* Agrobacterium-*infected tobacco leaf tissues*

Transient expression of the SUPER-*RiLUC* and SUPER-*RLUC* gene fusions was examined by means of agro-infusion of tobacco leaf tissues to see if the *RiLUC* reporter gene could generate a functional luciferase polypeptide in plant cells. Dual luciferase assays of *RLUC* and *RiLUC* normalized against a CaMV 35S–driven firefly luciferase gene (*FiLUC*) fusion showed similar mean ratios of luciferase activity (Table 1). Our transient data do not agree with previous

Table 1. Comparison of *RLUC* and *RiLUC* reporter genes by transient analysis and in stable transgenics.

	RLUC	***RiLUC***
Transient agro-infusion experiment (in vitro dual luciferase assay)		
Ratio of S&G to LARII	1.79±0.21	1.80±0.28
Stable transformant analysis (in vivo floating leaf disc assay)		
Average Relative Light Units-R_0	1783±337	1927±336
Average Relative Light Units-R_1	24927±8892	22681±5703

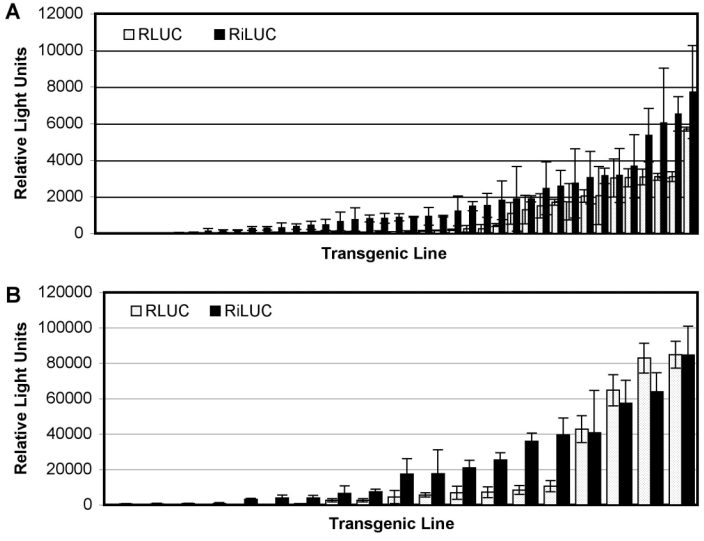

Figure 3. Luciferase activity of *RLUC* and *RiLUC* transgenic lines. Transformants were grown at 21°C under artificial illumination with a photoperiod of 16/8 h for 4 wk. Leaf discs from the second and third true leaves from 4-8 individual transformants were assayed in duplicate. Error bars represent standard errors. (A) Luciferase assays of 41 independent R_0 transformants. (B) Luciferase assays of 19 independent R_1 transformants.

observations that introducing the modified CAT-1 intron into the N-terminal coding region of the *GUS* reporter gene (Ohta et al., 1990) enhances transient activity in tobacco protoplasts. This is not surprising, considering that intron position within the coding region of the firefly luciferase reporter gene was reported to have marked effects on expression levels (Bourdon et al., 2001). Nevertheless, *RiLUC* luciferase activity was similar to that of *RLUC*, suggesting that the CAT-1 intron is being spliced correctly in tobacco leaf tissues, producing an active luciferase polypeptide.

Analysis of RLUC *and* RiLUC *in stably transformed tobacco*

Luciferase assays were performed on stable transformants harboring the *RLUC* and *RiLUC* reporter gene fusions to provide a more accurate analysis of luciferase activity levels and tissue specificities. As shown in Figure 3A, a similar dis-

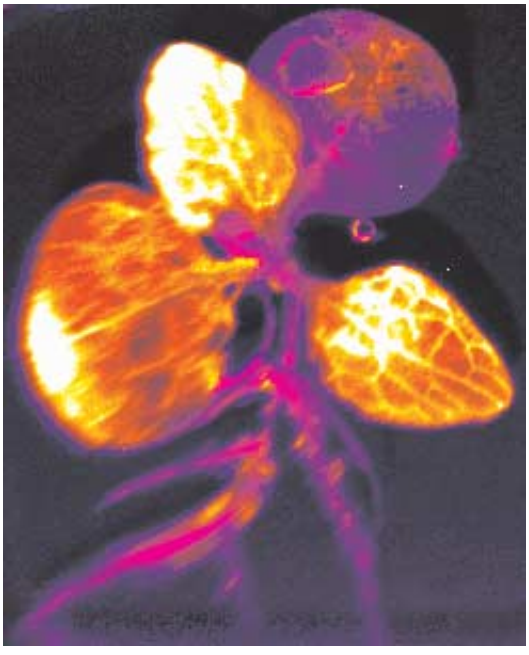

Figure 4. In vivo luciferase assay of a representative *RiLUC* transgenic line. Seeds were sowed on MS agar and grown at 21°C under artificial illumination with a photoperiod of 16/8 h for 2 wk. Seedlings showing their first set of true leaves were assayed for luciferase expression. The luminescent image was manipulated by using NIH Image software (v.1.63). Black represents no photons, and white indicates a saturation of photons.

tribution of luciferase activity from leaf tissues of 41 independent R_0 transformants containing either the *RLUC* or *RiLUC* fusions was observed. A similar distribution of luciferase activity was also observed when analyzing leaf tissues from R_1 transformants (Figure 3B). Average RLUs, calculated for *RLUC* and *RiLUC* transgenic lines (Table 1), support these observations. The *RiLUC* average is slightly higher than the *RLUC* average in the first generation (R_0), which is contradictory to results from the second generation (R_1). Nonetheless, all mean values from *RLUC* and *RiLUC* are within the range of the standard errors and are consistent with our transient analysis (Table 1). In vivo analysis of seedlings transformed with either reporter gene fusion showed similar patterns of tissue specificity. Luciferase expression was observed in leaves, cotyledons, roots, hypocotyls, petioles, and stems (Figure 4). It can be concluded from the data that introducing a portable intron into *RLUC* does not alter its properties.

In summary, we have generated a new luciferase reporter gene, *RiLUC*, which will be useful in detecting transgenic luciferase where contaminating bacteria is problematic. Engineering *RiLUC* has allowed development of a transient *Agrobacterium*-based dual luciferase assay, combining the sensitivity of the *RiLUC* and *FiLUC* reporter gene systems. The dual assay is useful in studying gene silencing mechanisms, as well as transcriptional and posttranscriptional regulatory processes.

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